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Effects of Pharmacologic Intervention on Oxygenation, Lung Water and Protein Leak in the Pseudomonas ARDS Porcine Model

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Almost twenty-five years after Ashbaugh and Petty published their report of an explosive form of lung injury in critically ill patients, which they termed adult respiratory distress syndrome (ARDS), the mortality and morbidity from this condition remains essentially unchanged. ARDS continues to present a formidable clinical challenge to the clinician, whether in civilian or military practice. Approximately 150,000 cases per year are seen in the United States and it frequently affects young previously healthy patients. ARDS represents an extremely complex sequela to shock, sepsis, civilian or military trauma and a number of other conditions, of widely varying etiology, which have a common clinical presentation and pathophysiology as ARDS.

The background to ARDS and the important role played by military medical personnel in first recognizing the association of severe trauma and infection with end-organ injury such as ARDS has been reviewed in a previous report from this laboratory and will not be discussed here.

RAII; Lab Animals; Pigs; Volunteers; Actue Respiratory Distress; Capillary Permeability; Swine

FOREWORD

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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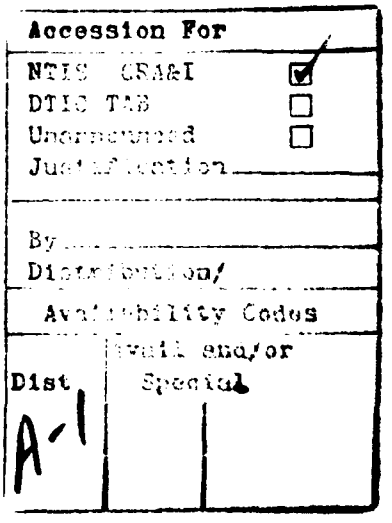

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Introduction

Almost twenty-five years after Ashbaugh and Petty published their report[1] of an explosive form of lung injury in critically ill patients, which they termed adult respiratory distress syndrome (ARDS), the mortality and morbidity from this condition remains essentially unchanged. ARDS continues to present a formidable clinical challenge to the clinician, whether in civilian or military practice. Approximately 150,0000 cases per year are seen in the United States and it frequently affects young previously healthy patients. ARDS represents an extremely complex sequela to shock, sepsis, civilian or military trauma and a number of other conditions, of widely varying etiology, which have a common clinical presentation and pathophysiology as ARDS. Although an extremely large number of conditions have been shown to predispose to the development of ARDS, many, such as fat embolism are associated with a relatively low mortality rate. Other causes of ARDS such as sepsis, require specific treatment. In the absence of such specific therapies, these are associated with an extremely high mortality rate. This association of sepsis and ARDS is of particular importance as together with trauma and aspiration these causes account for almost 75% of all cases of ARDS. Further, sepsis in the setting of ARDS either as a cause or complication carries a particularly high mortality. In recent years, with increased knowledge of the pathophysiology of acute lung injury and derangements in microvascular function and the role of complement, eicosanoids and cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and therapeutic intervention has begun to focus on inhibition or blockade at pivotal sites in the complement, eicosanoid and cytokine cascades, in an attempt to modify the host response to injury and attenuate or prevent end-organ damage such as that seen with

ARDS. The mortality associated with this condition remains at 40-60% and rises sharply in the presence of sepsis or additional organ failure. In addition to being a complication, sepsis is also the most frequent cause of ARDS and in this setting mortality rises to almost 90%. Treatment to date has been primarily supportive, consisting of ventilation, aggressive pulmonary toilet antibiotic therapy and when indicated, inotropic agents to support a compromised myocardium.

The background to ARDS and the important role played by military medical personnel in first recognizing the association of severe trauma and infection with end-organ injury such as ARDS has been reviewed in a previous annual report from this laboratory and will not be discussed here. The recent development of monoclonal antibodies to endotoxin and TNF-α, along with cytokine receptor antagonists, such as IL-1 receptor antagonist (IL-1ra) represents an exciting new advance in the endeavor to improve outcome in sepsis and organ failure such as ARDS.

The mechanisms that lead to the development of this protein rich inflammatory edema are extremely complex. Many substances have been implicated as either triggers or mediators of a cascade of events which, once started, spins rapidly out of control. Those substances implicated include endotoxin[2], complement[3], eicosanoids[4,5] and cytokines such as tumor necrosis factor-α[6,7] (TNF-α), interleukin-1[8] (IL-1), and interleukin-6[9]. It has long been speculated that, of the cells mediating the acute lung injury, the neutrophil is the one most central to the disease process[10-13]. This speculation is supported by the fact that neutrophils sequester in the lung, are capable of adhering to the endothelium and migrating from the vascular compartment to the interstitium and of producing many of the mediators

involved in the pathogenesis of ARDS. Furthermore neutrophils are found in greatly increased numbers in bronchoalveolar lavage samples from ARDS patients.

Three patterns of neutrophil-endothelial adherence have been described to date[13]. These are spontaneous neutrophil adherence, activated neutrophil adherence and activated endothelial adherence. Of these mechanisms, the first causes margination of neutrophils in the lung and is thought to be due to weak attractive forces generated by a divalent cation process. The physics of particulate flow also contribute to the marginating process whereby neutrophils are pushed out of the area of axial flow in the blood vessel in favor of the smaller erythrocytes. A dynamic equilibrium exists between those neutrophils in the circulation and those in the marginated pool.

The latter two patterns of neutrophil-endothelial adherence are active processes and lead to sequestration of neutrophils in the pulmonary vasculature. In contradistinction to margination, sequestration of neutrophils occurs due to strong adhesive forces generated between the neutrophil and endothelial cell, often mediated by glycoprotein adhesion molecules on the neutrophil and on the endothelial cell[14].

The strongest adhesion molecules on the surface of the neutrophil, called integrins, are collectively known as the CD11/CD18 adhesion receptor complex. These molecules are known to become active within a very short time of exposure to substances such as chemotactic peptides, activated complement fragments, leukotrienes and platelet activating factor. Adhesion complexes present on the endothelial cell surface tend to differ from those present on the neutrophil in that they take a period of hours, rather than minutes, to become activated and appear to respond to different stimuli such as thrombin and the cytokines,

TNF-α and IL-1. These receptors may promote neutrophil adhesion in the absence of activation of the CD11/CD18 receptor complex on the neutrophil.

It has been shown that this pulmonary leukosequestration is greatly increased in the presence of complement activation following cardiopulmonary bypass or renal hemodialysis[15]. In this setting, neutrophils actively attach themselves to the endothelium of the pulmonary vasculature by means of adhesion receptors. The outpouring of proteinases and toxic oxygen species that accompanies neutrophil activation occurs with the neutrophil tightly attached to the endothelium and thus, in a position to wreak greatest havoc on the endothelium as they migrate to the interstitium and ultimately the epithelial side of the alveolar-capillary membrane.

This hypothesis of acute lung injury holds that neutrophil activation is preceded by neutrophil adherence to endothelium and migration. Neutrophil activation is characterized by an increased respiratory burst, production of toxic oxygen products and activation of the hexose monophosphate shunt. The products of neutrophil activation are released into a protected microenvironment generated by the adhesion of the neutrophil to the endothelium, thus preventing the oxidant scavengers and proteases from being as effective as they might otherwise be. This results in increased local concentrations of toxic oxygen species and increased endothelial damage as depicted in Figure I. In this setting, neutrophil adhesion to

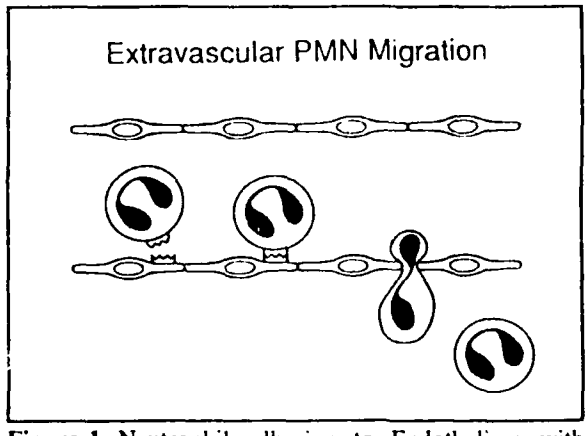

Figure 1 Neutrophil adhesion to Endothelium with subsequent migration

the endothelium is clearly a pivotal step. A growing body of evidence implicates blood PMNs as primary mediators of end organ damage associated with multisystem organ failure in sepsis, particularly the alveolar capillary membrane damage characteristic of sepsis-associated acute lung injury[16,17]. Using a porcine model of septic acute lung injury, our laboratory has previously correlated the appearance of TNFα in the circulation with priming of PMNs for toxic oxygen metabolite generation, increased expression of PMN $ß_2$ integrins and consequent loss of PMNs from the circulation[18]. *In vitro* studies indicate that TNFα activates PMNs for oxidant generation, phagocytosis, degranulation and adherence[19-21]. Though augmentation of these critical functions primarily prepares PMNs for first line immune defense, these functions are equally capable of precipitating host tissue damage.

In addition to oxygen free radicals, neutrophils and monocytes also release vasoactive substances such as eicosanoids and leukotrienes which directly affect pulmonary and systemic vascular tone. Along with alveolar macrophages, these cells also generate large amounts of cytokines such as TNF-α, IL-1 and IL-6. In recent years, research has shown that cytokine networks play a central role in the initiation and propagation of the physiologic and pathologic events seen in acute lung injury. One of the key mediators of cytokine production and release is endotoxin, a component of the cell wall of gram negative organisms. Many pathophysiological derangements associated with gram negative sepsis result from the release of endotoxin[22,23], the lipopolysaccharide (LPS) component of bacterial cell walls, into the circulation. One key role of LPS appears to be the initiation of a cascade of "communication proteins" elaborated and released by the reticulo-endothelial system[23]. These proteins or cytokines play an important role in inflammation, both by direct action on cells at sites of

infection and by trafficking other cells of the immune system, such as polymorphonuclear phagocytes (PMNs)[24]. Of the numerous cytokines now recognized, tumor necrosis factor-α (TNFα) has emerged in recent years as a critical chemical mediator of sepsis syndrome[25-27]. TNF-α is a 17 Kd peptide produced predominantly by members of the mononuclear phagocyte system in response to particulate and soluble inflammatory stimuli[9]. Transcription of the gene for TNFα proceeds rapidly following exposure to inflammatory stimuli, with resultant extracellular release occurring within 15 minutes[28]. The TNFα gene codes for a 233 amino acid protein which undergoes proteolytic cleavage of a 76 residue signal peptide leaving a 157 amino acid, active cytokine[6]. This secreted protein contains one intrachain disulfide bridge and exists as a dimer or trimer in circulation[29].

Significant clinical and experimental evidence implicates TNFα as central to the pathogenesis of septic shock. Elevated TNFα plasma levels are detected with greater frequency in septic patients[30] and plasma levels of TNFα correlate in some series with severity of illness and mortality rates[31]. Several animal and human studies show prompt surges of TNFα in circulation following intravenous injection of LPS[32,33], with metabolic and pathophysiological consequences which mimic gram negative septicemia[7]. Animals (e.g. C3HEJ mice) resistant to the effects of endotoxin, appear to have protection conferred by a genetic inability to manufacture native TNFα. These findings strongly suggest a pivotal role for TNFα in the evolution of septic shock from gram negative aerobic organisms.

In light of this evidence, we explored more closely the interaction between TNFα PMN activation in gram negative bacteremia. To accomplish this, we utilized a monoclonal antibody directed at biologically active, circulating TNFα. We sought to block the actions of

TNFα *in vivo*, thereby preventing PMN activation and attenuating lung injury associated with the porcine model of experimental sepsis. In light of the previously reported systemic effects of TNFα[34], we also predicted a modification of the hemodynamic derangements characteristic of experimental gram negative septicemia[35]. Our studies revealed significant protection against lung injury and altered hemodynamic performance in this model. Of striking interest however, was the discovery of conflicting effects on PMN function, with near complete inhibition of CD11/18 adhesion receptor expression but persistence of enhanced oxygen radical generation. We suggest that inhibition of adhesion receptor expression prevents the interaction of the activated PMN with pulmonary capillary endothelium, and therefore prevents toxic PMN metabolites from mediating alveolar capillary membrane injury. Further, by preventing both direct systemic actions of TNFα and PMN/endothelial interaction, monoclonal antibody to TNFα also inhibits the evolution of the cardiopulmonary derangements typical of this experimental model. The toxic effects of TNF-α *notwithstanding, sepsis syndrome represents the net result of interaction of many mediators,* non-cellular and cellular, of the host response to injury.

A number of other approaches have been employed in an effort to modify this host response to injury. These include the use of steroids, cyclooxygenase blockade or modulation of cytokine activity, employing cytokine receptor antagonists. Cyclooxygenase blockade with non-steroidal anti-inflammatory agents, blocks the neutrophil respiratory burst, preventing adherent neutrophils from generating large amounts of reactive oxygen species. In addition, these agents also block formation of vasoactive arachidonic acid metabolites which moderates the hemodynamic course of gram negative sepsis and attenuates sepsis-

induced lung injury[36,37]. Cytokine blockade using receptor antagonists[38] also blocks the widespread effects of cytokine excess seen in sepsis. Although these agents are of considerable benefit in moderating the host response to injury, neither moderates all of the features of the septic response in animal models. Ibuprofen, although attenuating both systemic and pulmonary sequelae of sepsis in the porcine septic model, does not prevent the characteristic neutropenia, or the late rise in PVRI or fall in p_aO_2[39]. Anti-TNFα monoclonal antibody also provides excellent hemodynamic protection and modifies neutrophil kinetics in gram negative sepsis, although it does not prevent release of arachidonic acid metabolites[34] known to be responsible for the early fall in p_aO_2 and marked rise in PVRI.

Altered vasoreactivity and maldistribution of blood flow are cardinal features of septic shock which historically have been attributed to direct effects of endotoxin or other endogenous lipid mediators released following activation of the cytokine network[40]. Surges of TNF-α in the circulation are observed in humans with sepsis syndrome[41], and in the porcine species following the onset of experimental *Pseudomonas aeruginosa* sepsis[42].

TNF-α and endotoxin promote release from vascular endothelium of Endothelium-Derived Relaxing Factor (EDRF) and Endothelin (ET), both potent mediators of vascular tone[43-45]. EDRF is now characterized as nitric oxide, and is produced in response to inflammatory and physical stimuli such as increased shear forces as occurs with enhanced blood flow during sepsis[46]. Endothelin is an endothelium derived peptide that produces sustained vascular contraction in porcine and human vasculature[47]. Kilbourne provided additional evidence linking TNF-α to endothelial production of vasoactive mediators by showing that TNF-α induced hypotension could be reversed by infusion of the EDRF

antagonist, N^G-monomethyl-l-arginine (LNMMA)[48]. EDRF represents but a single agent by which vascular endothelium regulates vessel tone. Endothelin-1 is a potent vasoconstrictor producing sustained hypertension when infused into humans[49]. Endothelin characteristically exhibits biphasic effects on vascular tone with vasodilator effects and hypotension as the predominant response to infusion of low concentrations. Vasodilator effects of endothelin in low concentrations is likely mediated by EDRF release[50]. EDRF can regulate ET release by negative feedback on ET release in response to chemical stimuli such as thrombin[51]. Likewise, EDRF antagonism in renal afferent arterioles potentiates the actions of endothelin[52]. Both EDRF and endothelin can modulate other potent vasopressors which regulate vessel tone (see below). Thus, regulation of EDRF and ET production is complex.

Refractory septic shock is characterized by lack of efficacy of catecholamines (e.g. norepinephrine, phenylephrine, and dopamine) as a therapeutic maneuver to moderate hypotension. This phenomenon may be due in part to excessive release of EDRF[53]. In view of the multiple interactions between EDRF and ET, it has become clear that both mediators must be studied simultaneously during a septic process.

In the complex paradigm of sepsis, vascular endothelium emerges as a regulator of immunological function and also serves as a rheostatic organ which modulates vasomotor tone. The present study examines circulating levels of nitrite (as a marker of EDRF release) and immunoreactive endothelin levels, during the course of gram negative septic shock and acute lung injury in pigs produced by infusion of live *Pseudomonas aeruginosa*. We hypothesized that altered vasoreactivity associated with gram negative sepsis may be linked to TNF-α surges in circulation, and that mechanisms controlling EDRF and endothelin release

by vascular endothelium differ. We studied the role of TNF-α in modulating release of EDRF and ET by infusing a monoclonal antibody to TNF-α. This protocol effectively removed the bioactivity of TNF-α during evolution of the septic process. The pretreatment dosing protocol used in these studies permits the pathogenic effects of TNF-α on circulating levels of EDRF and endothelin to be examined in concert.

The Model

The porcine model is used in all experiments. Swine weighing between 15-25 kgs are anesthetized with intramuscular ketamine hydrochloride 25 mg/kg and placed supine. Anesthesia is currently induced with sodium pentobarbital (10 mg/kg) and maintained with an infusion of pentobarbital and fentanyl at a rate of 5-10 mg/kg/min and 2.5-7.5μg/kg/min respectively. Previous studies were performed with paralyzed animals, since alveolar-capillary protein leak was measured with a computerized gamma camera in which the animals could not be allowed to move. Paralysis made evaluation of adequate anesthesia difficult. Following intubation with a cuffed endotracheal tube, the animals are ventilated with an F_IO_2 of 0.5 at a positive end expiratory pressure (PEEP) of 5 cm H_2O and a tidal volume of 12-15cc/kg at a rate to produce a $PaCO_2$ of approximately 40 torr at the beginning of the experiment.

Live *Pseudomonas aeruginosa* (PAO strain, 5 x 10^8 CFU/ml at 0.3 ml/20kg/min) is then administered for 1 hour. In pseudomonas (Ps) control animals this has been shown to produce a marked physiological deterioration, representative of acute ARDS, resulting in an immediate significant increase in pulmonary artery pressure (PAP) which persists throughout the entire duration of the experiment. Systemic arterial pressure (SAP) shows a progressive decline as does cardiac index (CI) and PaO_2. Extra-vascular lung water (EVLW) becomes significantly elevated when compared to

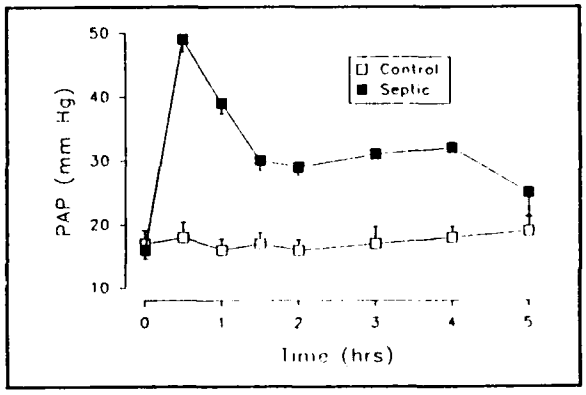

Figure 2 Changes in Pulmonary Arterial Pressure

saline controls.

Catheters are inserted into the left common carotid artery for monitoring of SAP and arterial blood gas determination, and into the right and left external jugular veins for infusion of *Pseudomonas* and the therapeutic agents to be studied. A Swan-Ganz catheter is passed from the right jugular vein into the pulmonary artery and wedged in a small branch with the balloon inflated, using pressure monitoring. It is thus possible to record pulmonary artery pressure (PAP), pulmonary capillary occlusion pressure (PCOP) and cardiac output, using a thermodilution technique. Cardiac output is converted to cardiac index (CI) by the formula:

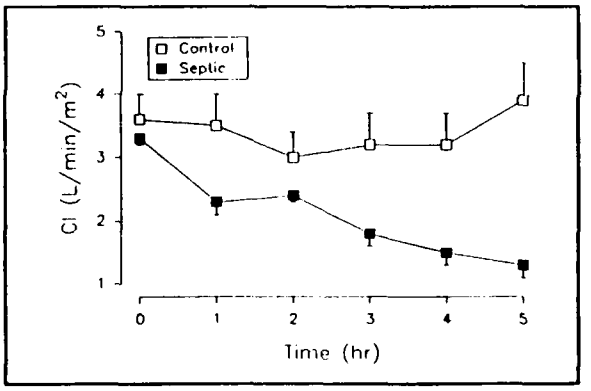

Figure 3 Changes in Cardiac Index

$$CI = \frac{CO}{0.112BW^{2/3}}$$

where BW is the body weight in kg. Arterial and mixed venous blood gases are analyzed using a blood gas analyzer (Instrumentation Laboratories, Model 113).

A 5 French femoral artery lung water catheter (American Edwards Laboratories, Model 96-020-5F) is passed into the lower abdominal aorta for measurement of extravascular lung water (EVLW) using a thermal dye dilution technique[54]. In this technique, 10 ml of iced, green dye solution (2 mg indocyanine green dye in 10 ml 5% dextrose) are injected as a bolus through the proximal port of the Swan-Ganz catheter as blood is simultaneously withdrawn through the thermistor-tipped femoral artery catheter connected to a densitometer

cuvette (Waters Instruments In., Model 402A) which is linked to a lung water computer (American Edwards Laboratories, Model 9310). The computer measures the mean transit times of the intravascular dye (MTD) and freely diffusible thermal component (MIT) as well as the cardiac output (CO). EVLW is calculated by the formula:

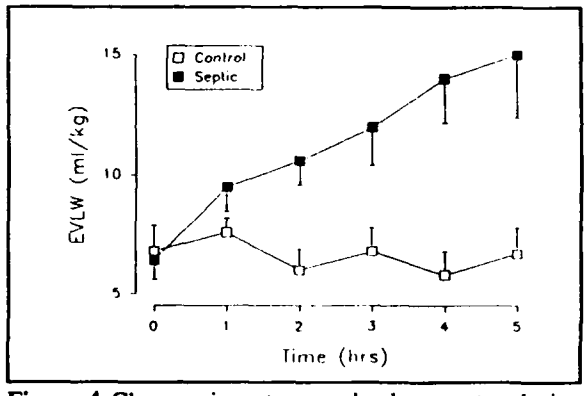

Figure 4 Changes in extravascular lung water during sepsis

$$EVLW = \frac{CO(MTD-MIT)}{BW}$$

Bronchoalveolar Lavage and Protein Assay

Using a fiberoptic bronchoscope (Machita VT-5100C, 4mm) bronchoalveolar lavage (BAL) is performed at 0 and 300 min. The middle and lower lobes of the right lung are lavaged (3 x 25 ml aliquots of sterile 0.9% NaCl in each lobe) at 0 min. This is repeated in the corresponding lobes of the left lung at 300 min. Lavage returns are consistently high (>75%) in all animals. BAL protein content, expressed as micrograms protein per milliliter of recovered lavage fluid, is performed on non-cellular fractions of the BAL by a modification of the Lowry technique[55].

Peripheral blood samples for isolation of neutrophils and subsequent assay of short and

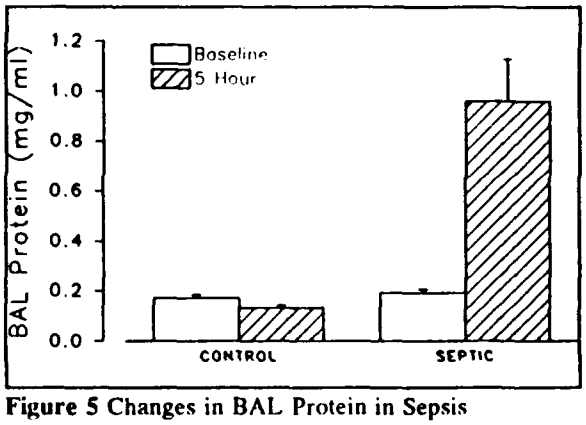

Figure 5 Changes in BAL Protein in Sepsis

long-lived toxic oxygen metabolites are withdrawn at baseline (zero timepoint), and at 5 hours (end-stage sepsis). The neutrophils are isolated using dextran sedimentation and Ficoll-sodium diatrizoate density gradient centrifugation. Assays of phorbol ester stimulated production of oxygen-dependent neutrophil products are performed as outlined in a previous report. Similarly, arterial blood samples are drawn at hourly intervals for estimation of neutrophil counts, thromboxane B_2 levels, TNF-α and IL-6 and also for measurement of CD18 receptor expression on peripheral neutrophils.

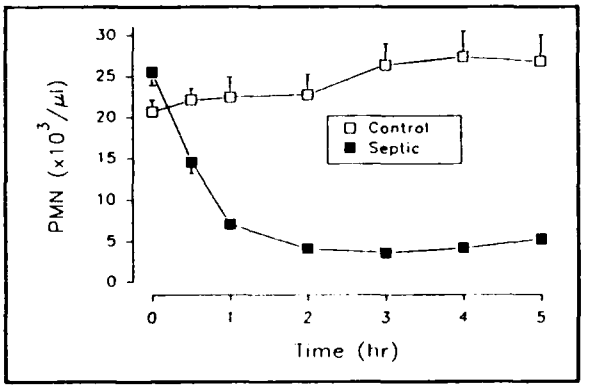

Figure 6 Effect of sepsis on peripheral neutrophil Count

Measurement of CD11/CD18 Receptor Expression

Saturating concentrations of fluorescein-conjugated monoclonal antibodies specific for the ß-subunit of the CD18 glycoprotein are incubated with the neutrophils after incubation with plasma (20 min at 4°C). To control for non-specific binding, the same concentration of fluorescein-conjugated murine IgG_{2a} is incubated with equally treated neutrophils. Cells are washed thoroughly and fixed in 1ml paraformaldehyde. Immunofluorescence intensity is analyzed with a flow cytometer equipped with a logarithmic amplifier[56]. The channel number (log scale) representing the

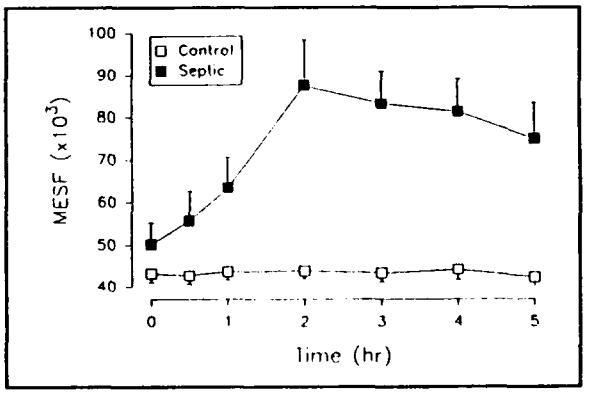

Figure 7 Effect of sepsis on CD11/CD18 adhesion receptor expression

peak fluorescence intensity of 5,000 cells is determined. Linear fluorescence-intensity is calculated from a logarithmic-linear calibration curve[57]. Quantitation of the expression of CD18 is calculated by subtracting the linear fluorescence intensity of the bound non-specific IgG_{2a} from the linear fluorescence intensity of the bound MoAb 60.3.

Tumor Necrosis Factor Activity

Arterial blood samples are collected at baseline, 30 min and then at 60 min intervals for measurement of plasma TNF activity. The mouse L929 fibroblast bioassay is used to quantify TNF activity[58]. Arterial blood samples are drawn into sterile glass tubes containing 0.15% EDTA and kept at 4°C. Specimens are centrifuged at 500 g for 20 min at 4°C and the resulting plasma frozen at -20°C until time of assay. L929 cells are seeded into flat-bottomed 96 well microtiter plates (Corning, NY) at a density of 4 x 10^4 cells/well and grown to confluence overnight in Dulbecco's minimal essential medium (GIBCO) containing 1% penicillin-streptomycin and 5% fetal calf serum (DMEM). Medium is then removed from confluent monolayers and 100 μl of DMEM containing Actinomycin-D (Merck, Sharp and Dohme, Westpoint, PA, final concentration = 5ug/ml) are added to each well. One hundred μl of each of the following are then added to selected duplicate wells containing L929 cells: 1) DMEM (0% cytotoxicity); 2) serial dilutions of recombinant TNF (5 x 10^3 to 6 x 10^{-4} U/ml) (Cetus Corp., Emeryville, CA); 3) plasma samples from different groups; 4)

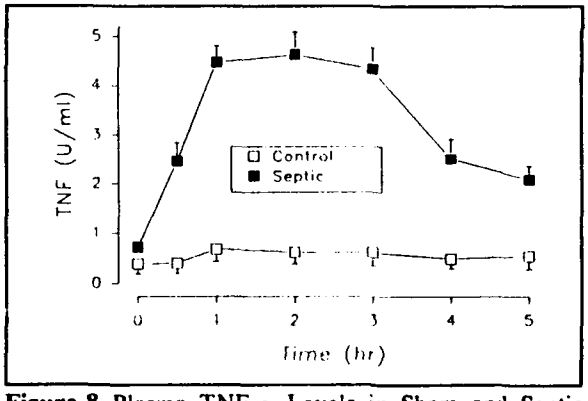

Figure 8 Plasma TNF-α Levels in Sham and Septic Animals

DMEM in blank wells without cells (100% cytotoxicity). Plates are then incubated for 20 hr at 37°C in 5% CO_2. Following incubation, the medium is removed and the L929 cells were stained for 10 min with 0.5% crystal violet in 20% methanol, rinsed in water and air dried. Optical density (OD) of each well was determined by a microplate reader (Bio-Tek EL 309) and calibrated to non-cellular reagent blanks at a wavelength of 550 nm. The percent cytotoxicity of L929 cells was calculated by:

$$\%Cytoxicity = \frac{OD\ wells\ with\ 0\%\ cytoxicity - OD\ experimental\ sample\ well}{OD\ wells\ with\ 0\%\ cytoxicity}$$

TNF activity is expressed in units per milliliter (U/ml), where one unit of TNF activity is defined as 50% L929 cytotoxicity.

Superoxide anion kinetics assay

Spontaneous and phorbol myristate acetate (PMA) stimulated generation of superoxide anion is measured in freshly harvested neutrophils, by continuously monitoring superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C[59,60] using a dual beam spectrophotometer (Shimadzu UV-160). Briefly, 650 μl of neutrophil suspension (4.0 x 10^6 cells/ml, in PBS) is added to a reaction mixture in a flat bottomed cuvette (Fisher Scientific). The reaction mixture contains 200μl of stock ferricytochrome C solution (16 mg/ml, Sigma chemical company) in a volume brought to 2.6 ml with PBS. Reference cuvettes contain cells plus cytochrome C plus

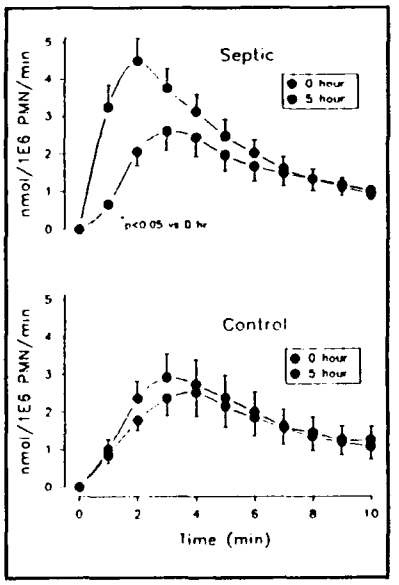

Figure 9 Superoxide production by PMN from septic and control animals

superoxide dismutase (100μg/ml). Cuvettes are permitted to equilibrate in the spectrophotometer at 37°C under continuous stirring for ten minutes. PMA (2.6 μl of 100 ng/ml stock) is then added to give a final cuvette concentration of 200 nmol/ml. The change in optical density is continuously recorded at 550 nm for a 10 minute period. Assays are performed in triplicate. The nanomolar extinction coefficient of 0.0211 for the reduction of ferricytochrome C is used to quantify superoxide anion production (24). Superoxide anion production, expressed as nmols of O_2^-/min/10^6 PMN, is calculated for each minute and plotted against time (Fig 9).

Experimental Design

TNF-α and Neutrophil Kinetics

Three groups of animals were studied. **Group I (Control, n=10)** received a 60 minute intravenous infusion of sterile saline. **Group II (Sepsis, n=10)** received a 60 minute intravenous infusion of live *Pseudomonas aeruginosa*, PAO strain ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). **Group III (Anti-TNFα, n=8)** were pretreated with monoclonal antibody to TNF-α (5 mg/kg I.V.) 15 minutes prior to an infusion of live bacteria similar to Group II animals.

Combined Cyclooxygenase-Cytokine Blockade

Three groups of animals were studied. Groups I and II received a 1 hour infusion of live *Pseudomonas aeruginosa* ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). There were 9 animals in Group I. Group II ($n=5$) received anti-TNFα, 5 mg/kg and ibuprofen 12.5 mg/kg, 15 minutes prior to the bacterial infusion and a further bolus of ibuprofen alone at 120 minutes into the study. Group III (n=11) received sterile saline only. All animals were studied for 300 min.

Nitric Oxide Levels in Sepsis

Three groups of animals were studied. The control group consisted of 5 animals. The septic group contained 7, and the antibody treated group (pretreatment with anti-TNF-α MoAb) contained 7 animals. To induce sepsis, animals were infused continuously for 1 hour with live *Pseudomonas aeruginosa* PAO strain ($5x10^8$ organisms/ml at 0.3ml/20 kg/min). Control animals received 1 hour infusion with an equivalent volume of sterile 0.9% NaCl.

Results

Plasma Tumor Necrosis Factor Activity

Plasma TNFα levels surged in Group II (Sepsis) animals within 60 min, reaching a peak of 4.54 ± 0.47 U/ml at 120 minutes, and remained significantly elevated over baseline and control values at 300 min. Group I (Controls) and Group III (Anti-TNFα) animals showed no significant increase in plasma TNFα activity throughout the study period (Figure 10).

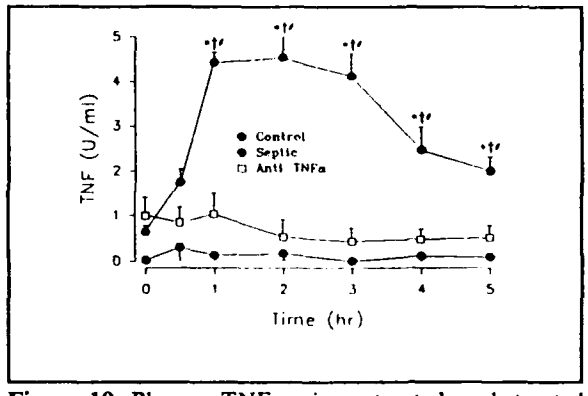

Figure 10 Plasma TNF-α in untreated and treated animals

Physiology of Porcine Sepsis and Effects of Anti-TNFα Antibody

Septic animals exhibited significant cardiopulmonary derangements following onset of sepsis. These derangements included early-phase pulmonary arterial hypertension, rapidly developing systemic arterial hypotension associated with significant deterioration of cardiac output. In the latter phases of the study period, septic animals failed to recover cardiac function and exhibited sustained pulmonary arterial hypertension, and systemic arterial hypotension, which was associated with an evolving metabolic acidosis and the development of significant arterial hypoxemia over the period of observation. In contrast, Animals treated with anti-TNFα showed significant improvement in many cardio-pulmonary derangements following the onset of *Pseudomonas* sepsis. After an initial decline in cardiac output during the 60 minute *Pseudomonas* infusion, these animals exhibited rapid recovery to baseline levels. The progressive systemic arterial hypotension and metabolic acidosis observed in

septic animals were not observed in anti-TNFα treated animals. Further, anti-TNFα pretreatment abolished the development of significant systemic arterial hypoxemia. However anti-TNFα failed to improve the early septic pulmonary arterial hypertension.

Bronchoalveolar Lavage Protein Analysis

The recovery of instilled BAL fluid at 0 min and 300 min was consistently high (≈ 70% return) and did not differ across groups. Baseline BAL protein content was similar in all three groups. In control animals, BAL protein content at 300 min did not differ from baseline (140 ± 18 vs 132 ± 21 μg/ml). In contrast, BAL protein content at 300 min in septic animals was more than 5-fold higher than baseline (770 ± 158 vs 137 ± 15 μg/ml, $p<0.05$). While anti-TNFα treated animals also showed an increase in BAL protein content at 300 minutes (313 ± 48 vs 141 ± 19 μg/ml), this was significantly less than that observed in septic animals (Figure 11).

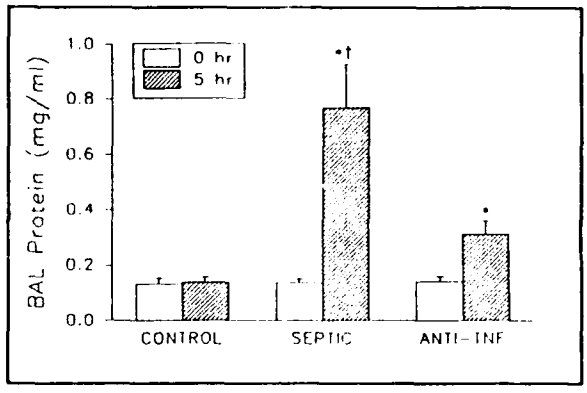

Figure 11 BAL protein in untreated and treated animals

Peripheral White Cell Counts

Septic animals became significantly neutropenic within 30 min, reaching a nadir at 120 - 180 minutes (Figure 12). Circulating white blood cell (WBC) counts fell by more than 80% and remained depressed throughout the study period. Pretreatment with anti-TNFα antibody altered the WBC profiles producing a biphasic response. Animals pretreated with anti-TNFα initially showed a significant drop in circulating WBC within 30 min, reaching a 50% reduction by 120 minutes of observation. However, from 120 minutes until completion

of the study, WBC counts rebounded to above baseline values (31.46 ± 2.15 vs 26.32 ± 1.35 $x10^3/\mu l$). Relative to other cells present in circulation neutrophilic populations accounted for the greatest decreases or increases in cell numbers in groups II and III respectively (data not shown).

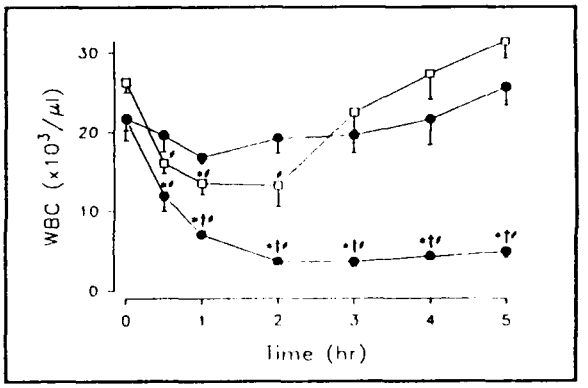

Figure 12 Peripheral white cell counts in untreated and treated animals

Circulating PMN morphology was monitored by examining cytocentrifuge preparations in animals from all groups during the 300 min period. Though total WBC counts differed significantly between septic animals and anti-TNFα treated animals at 300 min (Figure 12), the maturity of the PMN forms found in circulation did not. Equal numbers of immature PMNs (i.e., band forms, myelocytes) were observed between septic and antibody treated animals (data not shown).

Neutrophil CD11/CD18 Expression

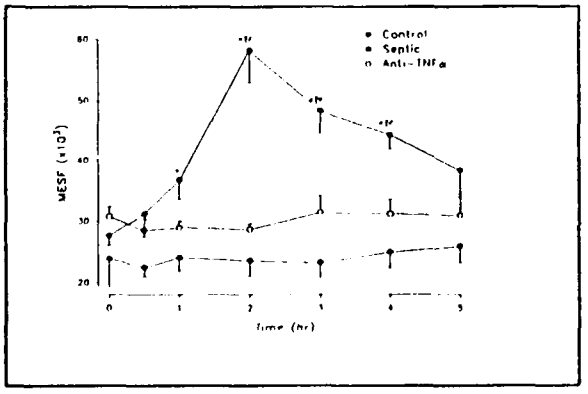

Figure 13 CD18 receptor expression in untreated and treated animals

PMNs obtained from septic animals exhibited significant upregulation of CD11/18 expression compared to baseline and control values (Figure 13). Peak values were observed from 120 to 240 min. In contrast, PMNs from control and pretreated animals showed no significant upregulation of CD11/18 expression over the course of study (Figure 13).

Lung Neutrophil Load

Myeloperoxidase content of lung tissue from animals in each study group was analyzed to assess lung PMN burden (Figure 14). Septic animals exhibited significantly higher myeloperxoidase content in lung tissue when compared to Group I (Control) animals (51.6 $\pm$ 9.9 vs 11.3 $\pm$ 2.8 U/g, $p < 0.001$). In pretreated animals, anti-TNFα antibody greatly reduced lung PMN burden when compared to septic animals (25.4 $\pm$ 3.3 vs 51.6 $\pm$ 9.9 U/g, $p < 0.05$). Thus, antibody treatment reduced lung PMN content despite an ongoing septic process.

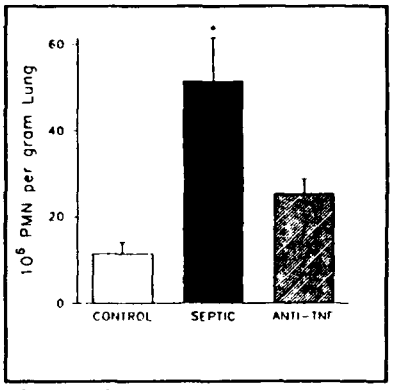

Figure 14 Lung neutrophil content in treated and untreated animals

Neutrophil Transendothelial Migration

PMN counts in recovered BAL lavage fluid, expressed as a percentage of the total recovered white cell count, were not significantly different between groups at time 0. In septic animals, lavage recovered significantly ($p < 0.05$) more PMNs at 300 min (24.5 $\pm$ 6.7 $p < 0.05$) than at 0 min (1.8 $\pm$ 0.4) and control animals, at 300 min (3.9 $\pm$ 1.4). There was no significant increase in PMNs recovered from BAL at time 300 (13.6 $\pm$ 6.5), compared to time 0 (4.7 $\pm$ 1.4) in pretreated animals.

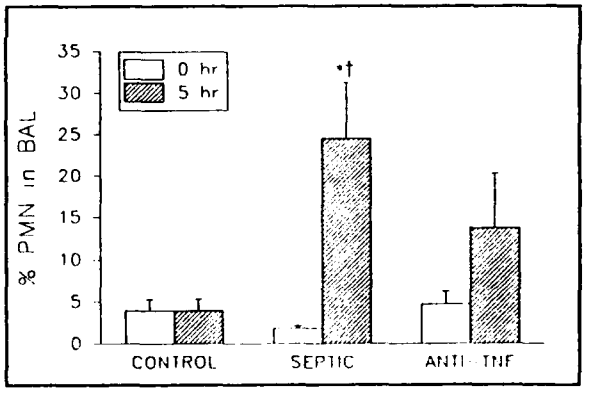

Figure 15 BAL neutrophil content in untreated and treated animals

Neutrophil Oxidant Production

PMNs obtained from septic animals at 300 min demonstrated a marked priming

response for PMA-stimulated O_2^- production when compared to baseline PMNs, as noted by an increase in both rate of production and peak production of O_2^-. (Figure 16). In contrast, PMNs from control animals showed no priming over the course of study. Pretreatment with anti-TNFα antibody failed to attenuate enhanced PMN short-lived oxidant generation. We found that PMNs obtained at 300 min from pretreated animals showed a similar degree of priming as that observed in septic animals.

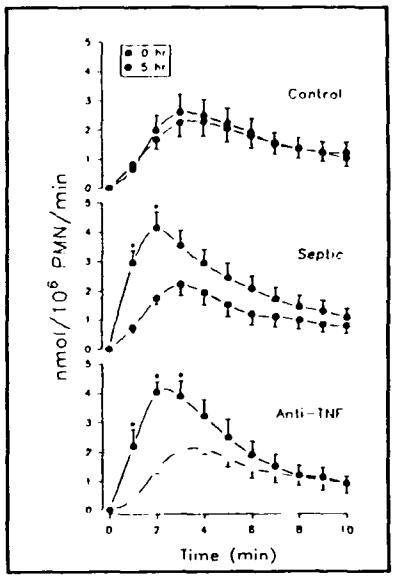

Figure 16 Neutrophil superoxide production in untreated and treated animals

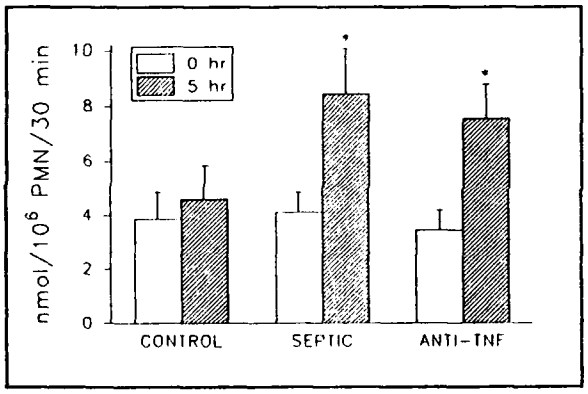

Figure 17 Neutrophil HOCl production in untreated and treated animals

Comparable findings were observed in PMN production of the long-lived oxidant, hypochlorous acid (HOCl). *Pseudomonas* sepsis resulted in significant priming of PMA-stimulated PMN HOCl production. This was not attenuated by pretreatment with anti-TNFα antibody (Figure 17).

COMBINED CYCLOOXYGENASE-CYTOKINE BLOCKADE

Physiology of Porcine Sepsis

Systemic vascular resistance index (SVRI) rose sharply with the onset of bacterial infusion, peaking at 30 minutes (4647±508 dyne-sec/cm^5/m^2 *vs* 2371±359 dyne-sec/cm^5/m^2,

0 min) in septic unprotected animals. SVRI subsequently returned to baseline at 60 minutes, where it remained until 180 minutes. It then showed a further moderate rise until the end of the study (3085±325 dyne-sec/cm^5/m^2, 300 min *vs* 2371±359 dyne-sec/cm^5/m^2, 0 min) (Figure 18). Pretreated animals showed no change in SVRI from baseline during the period of study and SVRI in this group was significantly less ($p < 0.05$) than that observed in septic animals during the *Ps* infusion (1960±97 dyne-sec/cm^5/m^2, group II at 30 min *vs* 4647±508 dyne-sec/cm^5/m^2, group I at 30 min).

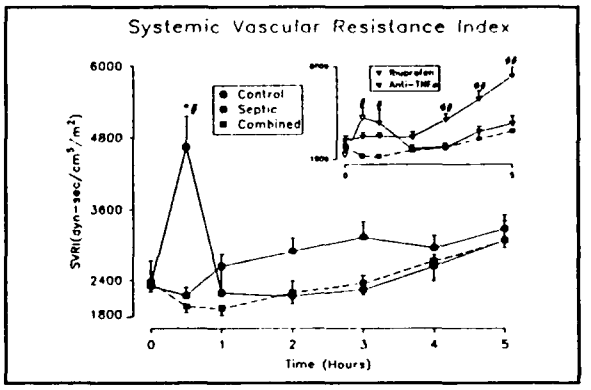

Figure 18 SVRI in combination therapy and with ibuprofen and anti-TNFα alone (inset)

Pulmonary vascular resistance index (PVRI) showed a response similar to that seen with SVRI (Figure 19). In group I, PVRI rose sharply with the onset of the bacterial infusion, peaking at 30 minutes (1563±249 dyne-sec/cm^5/m^2 at 30 min *vs* 256±39 dyne-sec/cm^5/m^2 at 0 min). It then briefly declined before rising again for the remainder of the study (Figure 19). Group II animals maintained PVRI at or near baseline levels (345±45 dyne-sec/cm^5/m^2, 300 min *vs* 167±47 dyne-sec/cm^5/m^2, 0 min) for the duration of study and values were significantly less than those observed in group I for the entire study period (345±45 dyne-sec/cm^5/m^2, 300 min *vs*

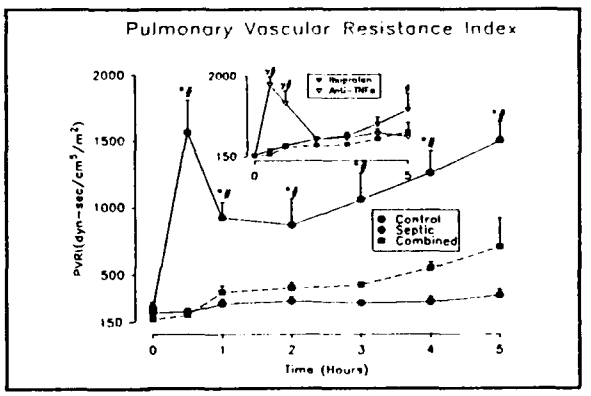

Figure 19 PVRI in combination therapy and with ibuprofen and anti-TNFα alone (inset)

1502±140 dyne-sec/cm^5/m^2, group I at 300 min).

Cardiac index (CI) fell precipitously in group I, during the first 30 minutes following onset of sepsis (2.3±0.2 L/min/m^2 at 30 min *vs* 3.5±0.2 L/min/m^2 at 0 min), recovered slightly, before resuming a sustained decline to the end of the study (1.6±0.2 L/min/m^2 at 300 min *vs* 3.5±0.2 L/min/m^2 at 0 min). Group II animals showed a moderate increase in cardiac index in the initial 60 minutes of study (4.1±0.1 L/min/m^2 at 60 min *vs* 3.3±0.3 L/min/m^2 at 0 min). CI subsequently declined for the remainder of the study period, but was not significantly different from group III animals (2.5±0.1 L/min/m^2, group I at 300 min *vs* 2.8±0.2 L/min/m^2, group III at 300 min).

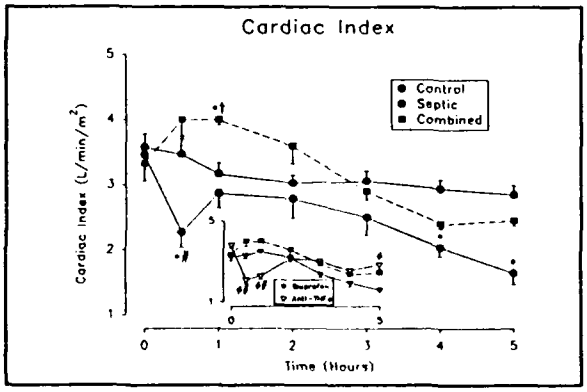

Figure 20 Cardiac index in combination therapy and with ibuprofen and anti-TNFα alone (inset)

Neutrophil Adhesion Receptor Expression and Kinetics

Neutrophil CD18 expression was studied at 60 min intervals. In group I animals, CD18 expression remained unchanged for the initial 60 min (Figure 21). Adhesion receptor expression subsequently rose sharply, peaking at 180 minutes (100±20 x 10^3 MESF *vs* 50±2.6 x 10^3 MESF, 0 min) before declining moderately prior to the end of the study. Neutrophil CD18 expression in group I was

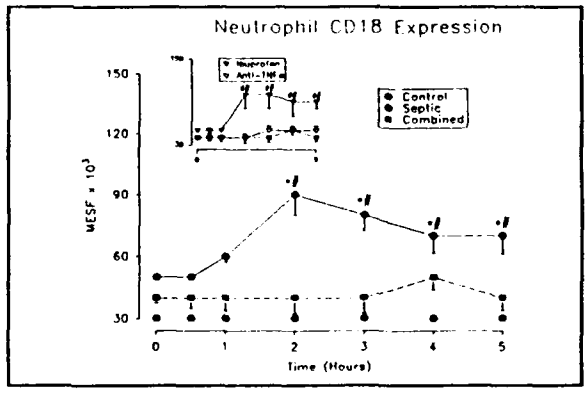

Figure 21 Neutrophil CD18 expression in combination therapy and with ibuprofen and anti-TNFα alone (inset)

significantly greater than that observed in group II from 120 minutes until the study conclusion (40±6.1 x 10^3 MESF, 300 min *vs* 90±10 x 10^3 MESF, group I at 300 min). There was no significant increase in neutrophil CD18 expression in group II throughout the study period (Figure 21).

Peripheral neutrophil count fell rapidly in group I following onset of sepsis, reaching a nadir by 120 minutes (3.6±0.3 x $10^3/\mu l$ at 120 min *vs* 24.5±1.3 x $10^3/\mu l$, 0 min) and remaining depressed for the remainder of the study coincident with the rise in neutrophil CD18 expression (Figure 22). Neutrophil counts in group II fell significantly from baseline in the first 60 minutes of study (13.8±3.8 x $10^3/\mu l$ at 60 min *vs* 24.3±1.4 x $10^3/\mu l$ at 0 min). Group II neutrophil counts remained at this level and were significantly greater than neutrophil counts observed in group I for the remainder of the study (12.4±4.0 x $10^3/\mu l$, group II at 300 min *vs* 3.8±0.5 x $10^3/\mu l$, group I at 300 min).

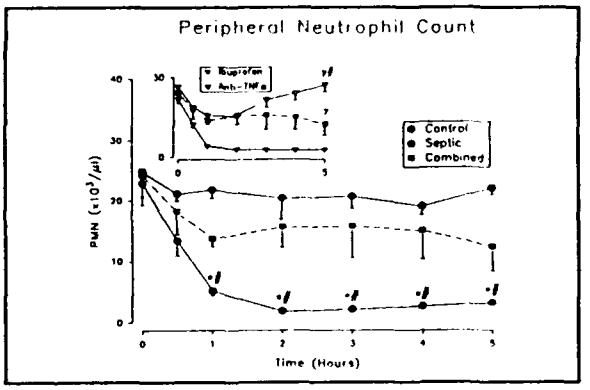

Figure 22 Peripheral white cell count in combination therapy and in ibuprofen and anti-TNFα alone (inset)

Coincident with the neutropenia observed, there was a marked increase in bronchoalveolar lavage (BAL) neutrophil content in group I at the study conclusion which was almost 5 times greater than that observed at the study outset (29±3 at 300 min *vs* 6±1% at baseline). There was marked attenuation of neutrophil migration in group II at the conclusion of the experiment (Figure 23). Neutrophil egress into the alveolar space was significantly less in group II than in group I at the study conclusion (10±5 %PMN, group II

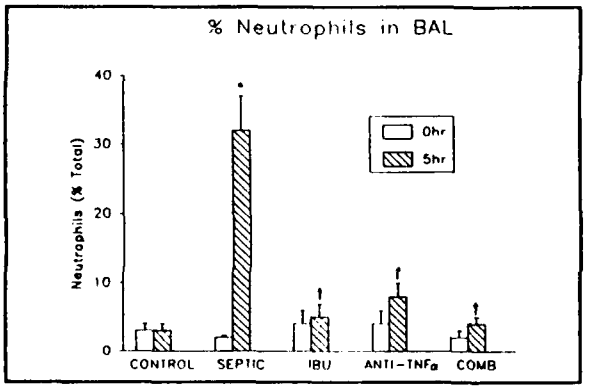

Figure 23 Neutrophil content in BAL in combination and monotherapy

at 300 min *vs* 29±3 %PMN, group I at 300 min).

Neutrophil Superoxide Generation

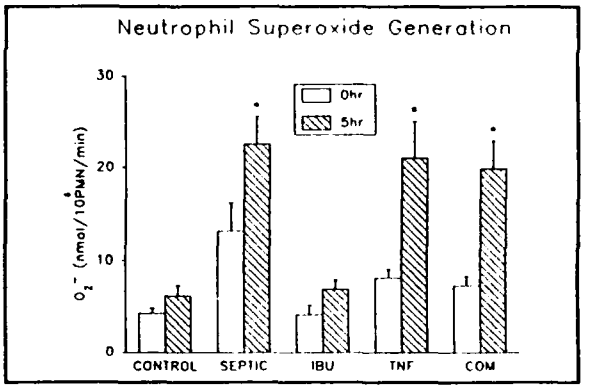

Figure 24 Neutrophil superoxide generation in combination and monotherapy

Neutrophils isolated from group I animals at the study conclusion showed evidence of significant priming for increased superoxide production over neutrophils isolated at baseline (22.5±3 nmol O_2^-/10^6 PMN/10 min at 300 min *vs* 13.2±3 nmol O_2^-/10^6 PMN/10 min at 0 min). Neutrophils isolated from group II animals were also primed for significantly increased superoxide anion production, compared with baseline neutrophils (19.9±3 nmol O_2^- /10^6 PMN/10 min at 300 min, group II *vs* 7.3 nmol O_2^-/10^6 PMN/10 min at 0 min, group II), (Figure 24). There was no difference in the degree of neutrophil priming at 300 min between groups I and II (19.9±3 nmol O_2^-/10^6 PMN/10 min, group II *vs* 22.5±3 nmol O_2^- /10^6 PMN/10 min, group I)

Oxygenation and Alveolar-Capillary Membrane Integrity

Arterial pO_2 (p_aO_2) fell sharply in group I animals following onset of the pseudomonas infusion (Figure 25). This fall was sustained throughout the study period (67±5 Torr at 300 min *vs* 241±10 Torr at 0 min). In animals receiving combined pretreatment (group II), p_aO_2 was maintained at baseline levels throughout the study period and did not differ from control

at any time (Figure 25).

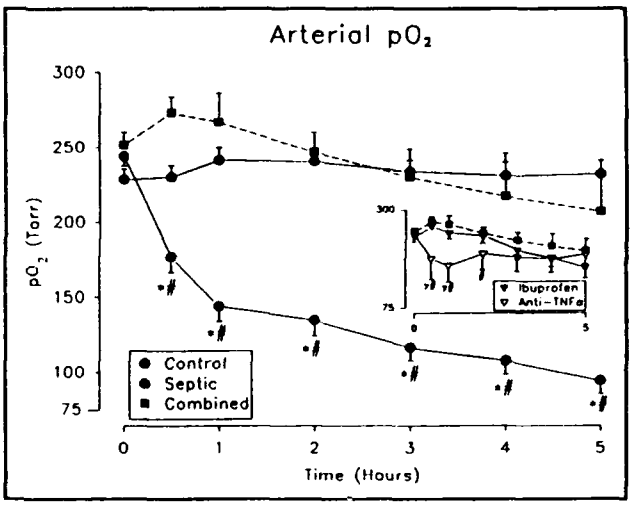

Figure 25 Arterial pO_2 in combination and monotherapy (inset)

A significant increase was observed in bronchoalveolar lavage protein content (BAL-P) in septic unprotected animals (group I) at 300 minutes. The BAL-P content in this group was almost 5 times greater than baseline in the same group (904 ± 123 μg/ml at 300 min *vs* 185 ± 14 μg/ml at baseline). BAL-P content in group II was not significantly different from baseline and was significantly less than BAL-P content in group I at 300 minutes (227 ± 44 μg/ml, group II at 300 min *vs* 904 ± 123 μg/ml, group I at 300 min).

NITRIC OXIDE

Hemodynamic Variables

Pretreatment with Anti-TNF-α MoAb significantly attenuated the decrease in systemic arterial pressure and the fall in cardiac index observed in septic animals. The nadir of systemic arterial pressures and SVR in septic animals coincided with peak levels nitrite. There were no significant differences in left ventricular preload (PCW) between groups throughout the study. Septic animals demonstrated a decrease in cardiac index especially in the mid and late period of the experiment. Animals that received the anti-TNF-α MoAb did not have significant decreases in CI in the mid and latter periods of the protocol.

TNF Activity

Septic animals demonstrated a rapid surge in plasma TNF activity that peaked at 2 hours (4.54 ± 0.47 u/ml at 2 hrs vs 0.64 ± 0.14 u/ml at zero hr) following the onset of sepsis and that returned to near baseline by 5 hours (2.00 ± 0.32 u/ml at 5 hours). Anti-TNF-α treated animals exhibited no elevation in plasma TNF activity, and were indistinguishable from the control animals (Figure 26).

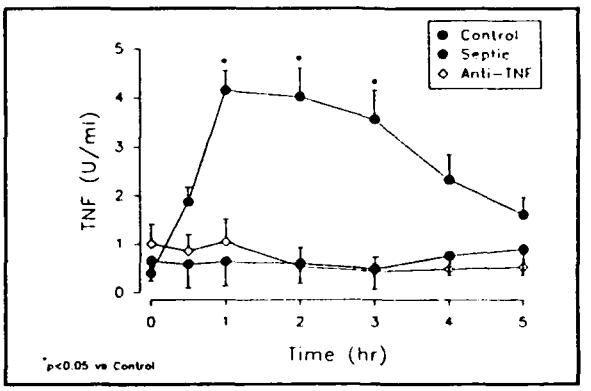

Figure 26 Plasma TNF-α levels in untreated and treated animals

EDRF Activity

Septic (untreated) animals displayed a rise in nitrite which peaked at 2 hours (0.454 ± 0.074 μM at 2 hrs vs 0.189 ± .094 μM at zero hr) into the study (Figure 27). Antibody treated animals showed significantly higher levels of nitrite (3.658 ± 1.75 μM at 1 hr vs 0.193 ± 0.10 μM at zero hr) with blood levels peaking 1 hour earlier than that observed in septic untreated animals. Antibody treated septic animals exhibited sustained, significant elevation in blood nitrite throughout the study when compared with both the septic and control groups (0.685 ± 0.356 μM at 5 hrs in anti-TNF group vs 0.218 ± 0.094 μM at 5 hrs in septic group vs 0.027 ± 0.017 μM at 5 hrs in control group). Nitrite levels in the *Pseudomonas* infusate and in the Anti-TNF-α MoAb

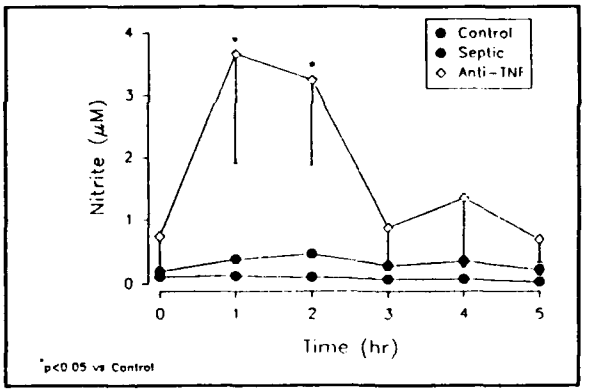

Figure 27 Plasma nitrite levels in untreated and treated animals

were negligible and did not contribute to elevated circulating nitrite.

Endothelin Activity

Septic animals exhibited a slow but significant rise in plasma endothelin levels which peaked at 3 hours (65.37 ± 28.32 pg/ml at 3 hrs vs 10.45 ± 1.82 pg/ml at zero hr) (Figure 28). Anti-TNF-α MoAB significantly attenuated the rise in plasma endothelin and significantly blunted the peak of endothelin at 3 hours (24.06 ± 6.84 pg/ml at 3 hrs in anti-TNF group vs 64.37 ± 28.32 pg/ml at 3 hrs in septic group). Control animals exhibited stable endothelin levels (13.57 ± 4.05 pg/ml at zero vs 10.88 ± 2.72 pg/ml at 5 hrs) which did not change significantly during the study.

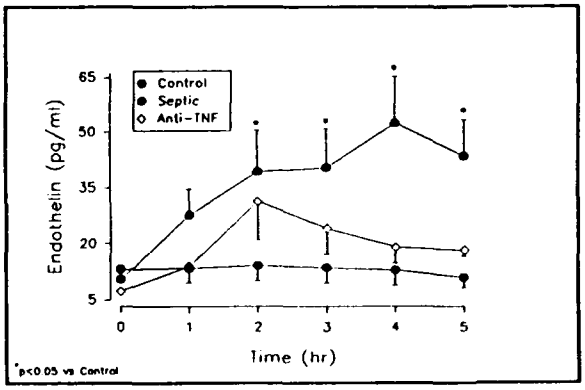

Figure 28 Plasma endothelin levels in untreated and treated animals

Future Studies

In Vivo

Neutrophil Kinetics

Future objectives involve continuing the characterization of PMN kinetics in this model with a view to identifying therapeutic regimens aimed at preventing the uncontrolled sequestration and activation of PMNs in response to sepsis while preserving their ability to phagocytose and kill invading pathogens. We plan to elucidate the cause of the acute drop in the PMN count seen in the first 30 min of sepsis. None of the treatment regimens we have studied have affected this abrupt early neutropenic phase. We wish to explore two hypotheses: either this is an adhesion receptor phenomenon or it represents the mechanical obstruction of the PMN in the pulmonary circulation. In terms of the adhesion receptors responsible, the LECAM-1/ELAM-1 complex would seem the most logical. We are currently engaged in studies to identify monoclonal antibodies to these and the ICAM-1 receptor that will cross-react in the porcine model. We plan to repeat the previous pretreatment studies using the antibodies above and anti-CD18b, with 60.3 and anti-TNF-α. In addition, the CD18 independent mechanisms of PMN adhesion seen later in the septic process will be investigated using these antibodies. The possibility that the early leukosequestration is as a result of cytoskeletal changes induced in the PMN has been suggested by other workers[61]. Preventing the increase in size and cytoviscosity of the PMN in response to endotoxin, has been shown to prevent the trapping by 5μm pores *in vitro* and to prevent lung leukosequestration in other animal models. Cytoskeletal manipulation can be achieved using drugs that stabilize the F-actin polymers such as phalloidin and antamanide[62],

or disrupt the F-actin polymers, such as cytochalasins[61,62], or manipulate the microtubule formation with vindesine. The effects of preincubating isolated PMN or pretreating the animals with these drugs, on the acute neutropenia following sepsis will be examined.

Posttreatment

Pretreatment studies provide vital information regarding the mechanisms by which various mediators and cells mediate their effects. In a clinical situation the efficacy of therapy given after establishing a diagnosis of sepsis-induced acute lung injury is of critical importance. We therefore plan to repeat the monoclonal studies, administering them after the onset of sepsis. We have previously shown post-treatment therapy, using ibuprofen to be of benefit in our model.

In Vitro

In parallel with the work we intend to carry out in vivo, we have designed a series of in vitro experiments that look more closely at aspects of our model in isolation. It is hoped that data obtained from this work in conjunction with the *in vitro* studies will provide a more complete understanding of the complex pathophysiology of acute lung injury.

Neutrophil Function

We have preliminary work looking at the function of the PMN in response to a variety of stimuli known to exist in our model. These PMN functions include reactive oxygen molecule (ROM) production, adhesion receptor expression, adherence and phagocytosis. The preliminary work concentrated on the effects of preincubation of isolated PMNs with TNF-α and IL-6, both alone and together. TNF-α is known to stimulate all of

the above parameters[20,63 65] and we have been able to confirm this in terms of ROM generation and adhesion receptor expression. Interestingly IL-6 failed to affect the adhesion receptor expression but did produce a marked ROM response, which was sustained over the study period, in contrast to the transient effect of TNF-α. We have been unable to show any synergism between these two cytokines, but the ability of the IL-6 to individually stimulate one PMN function in the absence of another requires additional study. We plan to look also at the effects of endotoxin (LPS) and interferon-gamma (IFN), in isolation and in combination.

Reports that adhesion is more than just the attachment of cells to endothelium and that it has profound effects on the function of the PMN and other cells, has prompted the design of another series of experiments to look at the effect of adhesion on ROM generation in the PMN. Using a modification of the adhesion assay we have previously defined and a microplate assay for ROM generation we will look at the effect of adherence and the effect of interrupting this adherence using monoclonal antibodies to a variety of adhesion receptors such as CD11/18, ICAM and ELAM, on PMN function, as well as the effect of TNF-α and IL-6 or ICAM and ELAM on PMN and endothelial expression respectively.

Cytokines

It has been postulated that the serum levels of cytokines we have measured in our model represent over-spill of locally produced paracrine mediators, initiated by the overwhelming response to sepsis[66]. During the normal inflammatory response, local release of these proteins or even membrane bound cytokines play the more dominant role. Reports that the cleavage of the extramembrane portion of the transmembrane TNF protein is

responsible for the 17 kilodalton protein we measure as TNF-α in our model[66] has prompted our search for these membrane bound cytokines in vitro. We have preliminary results, using immunofluorescent probes and the flow cytometer, that suggest the presence of a membrane bound TNF on the PMN from shocked animals, this work needs further elaboration and other cell lines such as the monocyte/macrophage lineage including the pulmonary intravascular macrophage need examination.

The receptors for cytokines have been the subject of a number of studies recently[67-70]. The current data support the concept that cytokines mediate their action via binding to cell surface receptors. We plan to examine the profile of cytokine receptors on the various cell lines and how these profiles change in response to sepsis in vitro and in our model, using immunofluorescent phenotyping as previously described, as well as evaluating protein synthesis and mRNA release using Northern blot analysis. It is anticipated that we will also be able to look at the effect of blocking the IL-1 receptor in our *in vivo* model.

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